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LOYOLA UNIVERSITY CHICAGO

TRANSFORMING GROWTH FACTOR BETA (TGF- $\beta$ ) SUPPRESSION OF CD8+ T CELL  
PROLIFERATION

A THESIS SUBMITTED TO  
THE FACULTY OF THE GRADUATE SCHOOL  
IN CANDIDACY FOR THE DEGREE OF  
MASTER OF SCIENCE

PROGRAM IN BIOCHEMISTRY AND MOLECULAR BIOLOGY

BY

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CHICAGO, ILLINOIS

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## ABSTRACT

Transforming Growth Factor Beta (TGF- $\beta$ ) is highly suppressive to both CD4+ and CD8+ T cell proliferation and function. In tumor microenvironments, TGF- $\beta$  has been described as immune suppressive, particularly to CD8+ T cells, however, the molecular mechanism behind how TGF- $\beta$  signaling controls T cell growth is not fully understood.

Here, we report that TGF- $\beta$  inhibits CD8+ T cell proliferation and reduces expression of the CARMA1/BCL10/MALT1 (CBM) signalosome complex in activated CD8+ T cells. The CBM signalosome is an essential scaffold that forms after T cell receptor (TCR) stimulation, leading to the activation of NF- $\kappa$ B and AP-1. This observed reduction in the CBM complex occurs simultaneously with a reduction in CD25 (IL-2R $\alpha$ ) expression. Together, the data suggest that TGF- $\beta$  inhibits antigen-stimulated CD8+ T cells by reducing CD25 expression and defines that the CBM complex may be one of the targets that mediates the effects of TGF- $\beta$  on CD25 expression.

## CHAPTER ONE

### INTRODUCTION

#### **Tumor Microenvironment**

The progression of tumor development due to its microenvironment was first postulated by German pathologist, Rudolf Virchow, in the 19<sup>th</sup> century. Although against conventional views at the time, Virchow identified the importance of the microenvironment in malignant cell survival and progression of tumor growth (David 1988). Virchow also identified a link between chronic inflammation and tumor progression, identifying the role of an immune response in cancer (Quail and Joyce 2013). The immune cell composition of the tumor microenvironment (TME) was not characterized until the mid-1970's, establishing the contribution of T cells, B cells, NK cells, and macrophages as having the capacity to infiltrate solid tumors (Witz 2009). It was then demonstrated in the 20<sup>th</sup> century that these infiltrating lymphocytes and proinflammatory cytokines were contributing to tumor progression and inflammation-linked cancer initiation (Nicolson 1988; Fidler 2003). Conversely, it has been well established that these infiltrating lymphocytes have impaired immune responses, demonstrating the complexity of the relationship between the TME and immune response (Witz 2009).

#### **Transforming Growth Factor Beta (TGF- $\beta$ )**

Transforming Growth Factor Beta (TGF- $\beta$ ) is a pleiotropic cytokine that regulates a variety of biological processes including proliferation, differentiation, migration, and immune



response (de Larco and Todara 1978; Sandford et al. 1997; Sanderson et al. 1995; Gorelik and Flavell 2000). This immunomodulatory role of TGF- $\beta$  was demonstrated in TGF- $\beta$ 1<sup>-/-</sup> mice where these mice develop chronic inflammatory disease and delayed wound healing (Böttinger et al. 1997). TGF- $\beta$  is involved in maintaining peripheral tolerance during homeostasis, regulating the function and survival of activated immune cells, and aids in resolving the immune response.

The TGF- $\beta$  superfamily includes growth factors, bone morphogenetic proteins, and activins. In mammals, TGF- $\beta$  contains three isoforms encoded by different genes, however TGF- $\beta$ 1 is the dominant isoform expressed by immune cells (Massagué 1998). TGF- $\beta$  is synthesized as a proprotein that is further processed into two separate mature proteins, TGF- $\beta$  and latency-associated protein (LAP), that noncovalently bind to form a complex, or the small latent complex (SLC) (Miyazono et al. 1988). The SLC can be secreted or be found in association with latent TGF- $\beta$ -binding protein (LTBP), which targets TGF- $\beta$  to the extracellular matrix (Rifkin 2004). The processing of TGF- $\beta$  to the active form disassociated from latent complex proteins is not completely understood, however, *in vitro*, changes in pH, temperature, or proteases can activate TGF- $\beta$  (Li et al. 2006).

TGF- $\beta$  has been characterized to regulate cellular function and differentiation in many cell types. In both epithelial cells and T cells, TGF- $\beta$  regulates cell cycle progression through the modulation of c-Myc expression, however the cyclin-dependent kinase inhibitors (CDKi) involved are cell type dependent (Frederick et al. 2004). TGF- $\beta$  has also been shown to block transcription of IL-2, a necessary growth factor for T cell proliferation (Li et al. 2006). In line with TGF- $\beta$ 's pleiotropic nature, it is also necessary for the polarization and differentiation of CD4<sup>+</sup> helper T (Th) cell Th17 and regulatory T (Treg) cell subsets, acting as both a suppressive

and stimulating cytokine of T cell proliferation and differentiation (Kehrl et al. 1986; Letterio and Roberts 1998; Li et al. 2006). Furthermore, TGF- $\beta$  has been shown to target and downregulate necessary molecules (IL-12R $\beta$ 2, Stat4) and transcription factors (GATA-3, T-bet) involved in signaling pathways that drive effector T cell differentiation, demonstrating its regulatory role in T cell differentiation (Gorelik et al. 2000; Heath et al. 2000). In addition to modulating CD4+ T cell activity, CD8+ T cell function and proliferation are also affected by TGF- $\beta$ . Upon antigen stimulation, CD8+ cytotoxic T cells produce cytolytic cytokines (perforin and granzyme B) and upregulate death receptor ligands (FasL) to mediate cell death of the target cell. TGF- $\beta$  has been shown to reduce granzyme B and perforin production, as well as reduce FasL expression resulting in reduced cytotoxic capability of CD8+ T cells (Genestier et al. 1999).

### **TGF- $\beta$ Signaling**

Immune cells express TGF- $\beta$  family transmembrane serine/threonine kinase receptors, TGF- $\beta$  receptor I and II (TGF- $\beta$ RI and TGF- $\beta$ RII respectively). Active TGF- $\beta$  binds to TGF- $\beta$ RII on the cell surface, which initiates the recruitment of TGF- $\beta$ RI and subsequent phosphorylation of Smad proteins (Ebner et al. 1993; Massagué 1998). Smad2, Smad3 and Smad4 are the primary mediators of TGF- $\beta$  signaling in T cells. Upon phosphorylation, Smad2 and Smad3 associate with common Smad4 (co-Smad4), and this complex can then translocate into the nucleus where it binds to Smad binding elements (SBEs) to facilitate their transcriptional regulation. Co-Smad4 facilitates nuclear translocation, however Smad2/3 complex can translocate independent of co-Smad4 (Massagué 1998). Furthermore, inhibitory Smad proteins 6 and 7 suppress TGF- $\beta$  signaling through competition for binding to the receptor or recruiting E3 ubiquitin ligases to degrade the receptor (Massagué 1998; Hata et al. 1998). These Smad complexes weakly bind to

DNA and primarily bind in association with a number of other transcription factors to control gene expression. Smad complexes control gene expression through the recruitment of histone modifying proteins such as histone-acetyl transferases (HAT) or histone-deacetylases (HDACs) to activate or repress target genes (Gaarenstroom and Hill 2014).

### **CD8+ T cells**

CD8+ T cells are an integral component of the adaptive immune system. CD8+ T cells, also known as cytotoxic T lymphocytes (CTLs) can respond to and target both pathogen-infected and tumor cells. CTLs mediate their effector function through pro-inflammatory cytokine production (type I IFNs, IFN $\gamma$ , TNF $\alpha$ ), granzyme and perforin production, and upregulation of death receptor ligands to mediate target cell death (Hashimoto et al. 2018). Naïve CD8+ T cells recognize antigen presented by antigen presenting cells (APCs) associated with major histocompatibility complex (MHC) class I molecule (Jiang et al. 1995; Garcia et al. 1996). As a result, the CD8+ T cells undergo rapid clonal expansion and become effector CD8+ T cells, mediating their cytotoxic activity against infected or tumor cells. Following clonal expansion and pathogen clearance, CTLs undergo a contraction phase, with 5-20% of antigen specific CD8+ T cells remaining as memory T cells that can self-renew independent of antigen stimulation (Wherry and Ahmed 2004).

CD8+ T cells can mediate immunosuppressive effects by killing CD4+ effector T cells or APCs (Jiang et al. 1995; Lanzavecchia et al. 1988; Simpson 1988). It has also been observed that CD8+ T cells can mediate immunosuppression through noncytolytic mechanisms (Salgame et al. 1989; Histasune et al. 1990; Koide and Engleman 1990; Hu et al. 1992). Similar to CD4+Foxp3+ Tregs, IL-2 and TGF- $\beta$  can induce and maintain CD8+Foxp3+ Tregs, however the CD8+ Treg

surface phenotype is not as well characterized as CD4<sup>+</sup> Tregs (Mayer et al. 2011; Chen et al. 2009). While CD8<sup>+</sup> Treg characterization and function are not well defined, the role of CD8<sup>+</sup> Tregs, specifically in the context of autoimmunity, are becoming increasingly important (Mayer et al. 2011).

Continuous antigen stimulation in chronic infection or cancer often results in T cell exhaustion. Exhaustion CD8<sup>+</sup> T cells were first described in the chronic lymphocytic choriomeningitis virus (LCMV) infection mouse model, revealing overexpression of inhibitory receptors, distinct epigenetic and transcriptional signatures, dysregulation in effector function, and poor proliferative capacity of virus specific CD8<sup>+</sup> T cells (Moskophidis et al. 1993). PD-1 is a co-inhibitory receptor, part of the CD28 family (Ishida et al. 1992). In tumor infiltrating CD8<sup>+</sup> T cells, TGF- $\beta$  has been shown to enhance the expression of PD-1 in a Smad3-dependent manner, suppressing T cell anti-tumor function (Park et al. 2016).

### **T cell Activation**

T cells express T cell receptors (TCRs) that recognize antigen peptides when presented on the surface of antigen presenting cells (APCs) in the context of a major histocompatibility complex (MHC) molecules. CD4<sup>+</sup> and CD8<sup>+</sup> T cell coreceptors recognize MHC class II and I molecules respectively (Bjorkman 1997). Upon antigen peptide recognition of the TCR presented by an MHC molecule, the TCR, in association with CD3 and zeta chains, are crosslinked, initiating the intracellular signal, forming an immune synapse (Gil et al 2002). Additionally, coreceptors CD4 or CD8 recognizing MHC class II or I molecules and colocalize to the immune synapse, recruiting Lck (lymphocyte-specific protein tyrosine kinase). The recruitment of Lck leads to the phosphorylation of immunoreceptor tyrosine-based activation

motifs (ITAMs) on the CD3 and zeta chain cytoplasmic tails (Shaw et al. 1990). The phosphorylated ITAM sequence creates a binding site of SH2 domain containing kinase, Zap70. Prior to TCR stimulation, Zap70 resides in the cytoplasm in an autoinhibited form and upon recruitment to the ITAMs, the inhibitory conformation is inhibited and phosphorylation by Lck stabilizes Zap70 for downstream TCR signaling events (Williams et al. 1998).

The recruitment of Zap70 to the immune synapse allows for the propagation of signaling events from TCR activation. Zap70 phosphorylates LAT (linker for activation of T cells) which acts as a signaling hub, leading to the recruitment of PLC- $\gamma$  (Phospholipase C gamma), adaptor molecules Grb2 (Growth factor receptor bound protein 2) and Gads (GRB2 related adaptor protein), and SOS (son Of sevenless homolog 1) and SLP-76 (lymphocyte cytosolic protein 2) that activate Ras, Rac, and Rho GTPases (Zhang et al. 1998). PLC- $\gamma$  generates secondary messengers 1,4,5-trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG), triggering the release of calcium that activates proteins including the transcription factor NFAT (Nuclear Factor of Activated T cells) (Brunvand et al. 1988; Gwack et al. 2007). DAG remains at the plasma membrane and activates protein kinase C (PKC) and RasGRP, leading to Ras-mediated activation of mitogen activated protein (MAP) kinase extracellular-signal-regulated kinase (ERK) and PKC- $\theta$ -mediated activation of NF $\kappa$ B (nuclear factor kappa B) (Putney 1987; Downward et al. 1990; Izquierdo et al. 1992). In addition to the activation of the LAT signalosome complex, TCR and CD28 signaling events activate PI3K (phosphatidylinositol-4,5-bisphosphate 3-kinase), leading to serine/threonine kinase AKT and mTOR activation, regulating growth and metabolism (Shim et al. 2011).

### **CARMA1-BCL10-MALT1 Signalosome Complex**

The CARMA1-BCL10-MALT1 (CBM) signalosome complex in T cells bridges TCR signaling events to canonical NF- $\kappa$ B activation and c-Jun N-terminal kinase (JNK) pathway activation (Hayden and Ghosh 2012; Egawa et al. 2003; Hara et al. 2003). The transcription factors NF- $\kappa$ B and AP-1 are critical for T cell activation, proliferation, and differentiation, as they regulate expression of genes necessary for these processes (Jung et al. 1995). Canonical NF- $\kappa$ B activation in T cells is triggered by TCR stimulation, leading to the activation of PKC- $\theta$ , subsequent CBM signalosome complex assembly, and activation of NF- $\kappa$ B through the degradation of inhibitory molecules (e.g. I $\kappa$ B $\alpha$ ) in the cytosol (Sun et al. 2000; Huang and Miyamoto 2001). I $\kappa$ B $\alpha$  is phosphorylated by activated I $\kappa$ B kinase complex (IKK $\alpha$ , IKK $\beta$  and NEMO/IKK $\gamma$ ) and subsequently degraded by the proteasome, releasing NF- $\kappa$ B for nuclear translocation (Baeuerle and Henkel 1994). Additionally, JNK signaling leads to nuclear accumulation of c-Jun and consequently AP-1 activation (Fuchs et al. 1996). JNK activation is achieved through the sequential phosphorylation of MAP kinases, however transforming growth factor beta-activated kinase 1 (TAK1) can also be recruited to the CBM signalosome complex and phosphorylation of JNK leads to subsequent c-Jun nuclear accumulation (Sakurai et al. 2002).

CARMA1 belongs to a family of membrane associated guanylate kinase (MAGUK) containing caspase recruitment domain (CARD) proteins including CARMA1 (CARD11), CARMA2 (CARD14), and CARMA3 (CARD10); CARMA1 is the primary isoform in lymphocytes (Bertin et al. 2001; Dimitratios et al. 1999; Gaide et al. 2001). In addition to the CARD domain, CARMA1 also consists of a coiled-coil (CC) domain, and PDZ, SH3, and GUK regions necessary for localization and cluster formation (Gaide et al. 2001). Upon phosphorylation by PKC- $\theta$ , CARMA1

oligomerizes via its CC domain, initiating the assembly of the CBM signalosome complex at the plasma membrane (Sun et al 2000). CARMA1 interacts with BCL10/MALT1 via CARD-CARD interactions, facilitating the formation of BCL10/MALT1 filaments and the recruitment and activation of NF- $\kappa$ B and JNK (Bertin et al. 2001; Gaide et al. 2001; Sakurai et al. 2002).

BCL10 (B-cell lymphoma/leukemia 10) is an adaptor molecule, constitutively associated with MALT1 (mucosa-associated lymphoid tissue lymphoma translocation protein 1). BCL10 interacts with CARMA1 via CARD-CARD interactions, and additionally contains a serine/threonine (S/T)-rich region at the C-terminus that is targeted for post-translational modifications (Zhang et al. 1999; Lobry et al. 2007). BCL10 constitutively associates with MALT1 via the Immunoglobulin-like (Ig) domains 1 and 2 (Ig1 and Ig2) of MALT1 and the C-terminus of BCL10 (Güet and Vito 2000). Consequently, phosphorylation of this S/T-rich site by IKK $\beta$  acts as a negative feedback mechanism by disturbing the interaction of BCL10 with MALT1, impairing NF- $\kappa$ B signaling downstream of TCR stimulation (Lobry et al. 2007). BCL10 can also be targeted for degradation by phosphorylation of Ser138 by CamKII (calcium/calmodulin dependent protein kinase II), resulting in decreased NF- $\kappa$ B signaling (Oruganti et al. 2011). BCL10 can also be regulated by ubiquitination as disassembly is just as critical in keeping T cell activity and function under control. The phosphorylation of Thr81 and Ser85 in the CARD region of BCL10 by IKK $\beta$  promotes the ubiquitination and subsequent proteasomal degradation of BCL10 (Lobry et al. 2007; Oruganti et al. 2011). As a result, the termination of CBM complex signaling to prevent prolonged NF- $\kappa$ B-mediated T cell survival is largely controlled by BCL10 phosphorylation and ubiquitination.

MALT1 constitutively associates with BCL10 and contains both scaffold and protease activity consisting of a N-terminal death domain (DD), three Ig domains, and a caspase-like (paracaspase) domain (Uren et al. 2000). MALT1 caspase activity is necessary for downstream NF- $\kappa$ B signaling, however the complete mechanism is not fully elucidated. It has been suggested that MALT1 auto-cleavage activity allows for release from the auto-inhibitory conformation, resulting in conformational rearrangement for binding and downstream signaling to occur (Hachmann et al. 2012; Wiesmann et al. 2012). MALT1 also contains predicted binding sites for the E3 ubiquitin ligase TRAF6 (tumor-necrosis factor associated receptor associated factor 6), which contributes to TCR-mediated NF- $\kappa$ B activation (Sun et al. 2004). Although the function of MALT1 is not completely understood, the scaffold function and protease activity are vital for efficient CBM assembly and downstream signaling.

### **Interleukin (IL)-2 Signaling**

The T cell growth factor, IL-2 was discovered in 1976 to support long term in vitro cell growth (Morgan et al. 1976). IL-2 transcription is upregulated upon TCR stimulation and is primarily produced by CD4<sup>+</sup> T cells. Among TCR induced transcription factors NFAT, NF- $\kappa$ B, and AP-1, IL-2 is also transcriptionally regulated by OCT1 (POU class 2 homeobox 1), HMGA1 (high mobility group protein), FOXP3 (forkhead box P3), and CD28 response elements (CD28RE) (Müller and Rao 2010; Kim et al. 2006; Spolski et al. 2018). In addition to inducing FOXP3 expression, TGF- $\beta$  has also been shown to block the transcription of IL-2 resulting in cell cycle arrest (Brabletz et al. 1993; Das and Levine 2008). IL-2 is necessary for naïve CD4<sup>+</sup> T cell differentiation into Th1, Th2, Th9, Treg, and T<sub>FH</sub> effect T cell subsets, promoting differentiation and survival of these cells. Conversely, IL-2 is inhibitory for Th17 differentiation, but promotes



their proliferative capacity (Spolski et al. 2018). IL-2 is critical for Treg development and suppressive function in maintaining immune homeostasis (Setoguchi et al. 2005). Additionally, IL-2 stimulates CD8+ T cell expansion after encountering antigen, it also regulates their differentiation into effector or memory phenotypes (Kalia et al. 2010). CD4+ and CD8+ T cells, among other cell types, express high affinity IL-2 receptors that initiates a specific response upon interaction with IL-2. Both CD4+ and CD8+ T cell respond to IL-2, however part of the response is regulated by an intricate network of transcription factors and the transcriptional regulation of central cytokine receptors that are subset specific (Malek and Castro 2010).

The IL-2 receptor is a trimer complex composed of the common gamma chain ( $\gamma_c$ ), IL-2R $\beta$  (CD122), and IL2-R $\alpha$  (CD25) (Leonard et al. 1984; Hatakeyama et al. 1989; Russell et al. 1993). The  $\gamma_c$  is the shared signaling component of the IL-2, IL-4, IL-7, and IL-21 receptors and CD122 contributes to both the IL-2 and IL15 receptors (Hatakeyama et al. 1989). CD25 is specific for IL-2 and is the high affinity component of the IL-2 receptor trimer, however, does not have any intracellular signaling capacity (Leonard et al. 1984). Upon interaction with IL-2, the IL-2 receptor complex activates the Janus kinase (JAK-STAT), ERK, and PI3K pathways. Canonical signaling primarily leads to the activation of STAT5, STAT1, and STAT3, which translocate into the nucleus to regulate transcription of target genes (Liao and Leonard 2013). The expression of IL-2 and CD25 are both under transcriptional control of STAT5, TCR induced (NFAT and NF- $\kappa$ B), and additional cytokine (IL-7, IL-12, IL-15, TGF- $\beta$ , and TNF) induced transcription factors, which serve as a feed forward loop between IL-2 production and IL-2 receptor signaling (Lin et al. 2012; Kim et al. 2006).

### Interleukin (IL)-4 Signaling

The type 2 immune response cytokine, IL-4, was first cloned in 1986 and identified as an inducer of immunoglobulin (Ig)E production and supported B cell growth (Noma et al. 1986). IL-4 is a classic Th2 cytokine, produced in response to allergy, asthma, and Th2 inflammation (Kopf et al. 1993). Similar to IL-4, IL-13 is also a mediator of Th2 inflammation and supports B cell proliferation and Ig production. IL-4 and IL-13 share transcriptional regulatory elements, and along with IL-5, are produced by CD4<sup>+</sup> T cells, basophils, eosinophils, mast cells, NKT cells, and ILC2 cells. However, production of IL-4 and IL-13 are differentially controlled among cell types (Lee et al. 2004).

IL-4 signals through two receptor complexes. The type I IL-4 receptor includes the IL-4R $\alpha$  (CD124) and  $\gamma_c$ , primarily expressed by lymphocytes, and the type II IL-4 receptor includes CD124 and IL-13R $\alpha$ 1 (CD213a1), predominantly expressed by epithelial cells (Junttila 2018). In T cells, upon the interaction between IL-4 and the IL-4 type I receptor, JAK/STAT signaling is activated, leading to the subsequent activation and nuclear translocation of STAT6 to regulate expression of target genes (Kotanides et al. 1995). STAT6-independent activation of PI3K and downstream mTOR activation is also regulated by IL-4 type I receptor signaling (Coffer et al. 1998).

CD8<sup>+</sup> T cells in the presence of IL-4 have been shown to induce CTLs that have phenotypic overlap with Th2 effector cells. The alternative CTL subset (Tc2) produces IL-5, IL-13, and IL-4, but has a lower cytotoxic capability compared to conventional CTLs (Croft et al. 1994 and Ranasinghe et al. 2007). In the context of viral infections, IL-4 and IL-13 have also been associated with decreased frequency of virus-specific CD8<sup>+</sup> T cells (Ranasinghe and Ramshaw

2009). Therefore conventionally, CD8<sup>+</sup> T cell response to IL-4 has been associated with decreased cytotoxicity and dampened antiviral immunity.

## CHAPTER TWO

### MATERIALS AND METHODS

#### **Mice**

C57BL/6J mice were purchased from Jackson Laboratory. 4 to 6 week old male mice were used for all experiments. All procedures were approved and monitored by the Institutional Animal Care and Use Committee of Loyola University Chicago.

#### **Cell Culture**

Cells were cultured in RPMI 1640 medium (GE Healthcare Hyclone) supplemented with 10% fetal bovine serum (Gemini Bioproducts LLC), L-glutamin (Hyclone), 10% solution of penicillin/streptomycin (Hyclone), 1mM sodium pyruvate (Corning), 0.1 M hepes (Corning), non-essential amino acids (Gibco), essential amino acids (Corning), and 50  $\mu$ M 2-ME (Fisher Scientific). Cells were maintained in the presence of 5% CO<sub>2</sub> at 37°C.

#### ***In vitro* T cell Activation**

Total T cells were collected via panning by incubating splenocytes on a 10 mm petri dish coated with goat anti-mouse IgG and IgM for 15 minutes at 37°C. Non-adhered T cells were carefully collected and maintained in RPMI 1640 (as described above). Total CD4<sup>+</sup> or CD8<sup>+</sup> T cells were purified from total splenocytes by negative selection via magnetic cell sorting (Bio Legend). Cells were activated by plate-bound anti-CD3 (5 ng/mL) and soluble anti-CD28 (1 ng/mL) when indicated in the presence or absence of additional recombinant cytokines at 37°C

for indicated durations. Recombinant cytokines include IL-2 (human; 10 ng/mL), IL-4 (mouse; 10 ng/mL), and TGF- $\beta$  (human; 2.5 ng/mL).

### **Flow Cytometry Staining**

Cells were stimulated as described previously for indicated times in a 96-well or 48-well flat-bottom non-tissue culture plate. For surface staining, cells were PBS washed, stained 10 min at RT with Live/Dead Zombie Aqua (BioLegend), PBS washed, and stained 10 min on ice with TruStain FcX anti-mouse CD16/32 (BioLegend). The following fluorochrome-conjugated antibodies were used: CD4 (GK1.5) APC-Cy7 (BioLegend), CD8 (53-6.7) FITC (BioLegend), CD25 (PC61) APC (BioLegend). Surface stained was done for 30 min on ice.

### **Western Blotting**

Cell lysates were collected directly in Laemmli buffer (2% SDS, 125 mM DTT, 10% glycerol, 62.5 mM Tris-HCl (pH 6.8)) or Triton X-100 buffer (10 mM phosphate puffer, 150 mM NaCl, 0.1% SDS) with protease inhibitors, followed by SDS-PAGE, transfer to PVDF membrane, blocking (5% milk in TBS/Tween), incubation (16h, 4°C) with primary antibody (1:1000), followed by incubation (1h, RT) with secondary anti-rabbit HRP or anti-mouse HRP (1:2000; Cell Signaling). Visualization was with ECL reagent (ThermoFischer) and Amersham Hyperfilm ECL (GE). The following primary antibodies were used: GRB2 (C-7) (Santa Cruz Biotechnology), BCL10 (331.3) (Santa Cruz Biotechnology), MALT 1 (D1) (Santa Cruz Biotechnology),  $\beta$ -actin (AC-15) (Sigma-Aldrich), CARMA1/CARD11 (A-4) (Santa Cruz Biotechnology), PKC- $\theta$  (C-18) (Santa Cruz Biotechnology).

### qPCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen). To quantify genes of interest expression levels, equal amounts of cDNA were synthesized using the RT<sup>2</sup> HT First Strand Kit (Qiagen) and mixed with the RT<sup>2</sup> SYBR Green qPCR Mastermix (Qiagen) and RNA was isolated from cells using the RNeasy Mini Kit (Qiagen) and converted to cDNA using the RT<sup>2</sup> HT First Strand Kit (Qiagen). mRNA concentration was normalized before cDNA conversation. qPCR was conducted at 95°C for 10 min, followed by 39 more cycles of 95°C for 15 s and 60°C for 1 min. The specificity of the reaction was verified by melt curve analysis. 18s rRNA was amplified as an internal control. The following primers were used:

CARMA1 FWD 5'- GTT CCA CGG CTC TTG TGT ATA G -3'

CARMA1 RV 5'- TGC ATT CCA CGT TAT CCC ATA G -3'

BCL10 FWD 5'- GTG TCCCAC TCC TTA GTT CTG -3'

BCL10 RV 5'- GCT GGC AGG GTG AGT ATA AA -3'

MALT1 FWD 5'- GTA GAA GTA ATG TGC CCG TAG AG -3'

MALT1 RV 5'-TCA TCA CTG CCC AGC TTA AAT A -3'

18s rRNA FWD 5'- CAT GGC CGT TCT TAG TTG GT -3'

18s rRNA RV 5'- CGC TGA GCC AGT CAG TGT AG -3'

## CHAPTER THREE

### TRANSFORMING GROWTH FACTOR BETA (TGF- $\beta$ )-MEDIATED MECHANISM OF SUPPRESSION IN CD8+ T CELL PROLIFERATION

#### Introduction

T cell activation and differentiation requires at least three signals. The T cell receptor (TCR) must recognize an antigen presented by an antigen presenting cell (APC) in the context of a major histocompatibility complex (MHC) molecule, which CD4 or CD8 co-receptors recognize MHC class II or I molecules respectively (Bonney-Berard et al. 1992; Biddison et al. 1982; Swain SL. 1983). The second signal is provided by co-stimulation through the interaction between CD28 on naïve T cells and CD80/CD86 surface expression on APCs (June et al. 1987). Third, cytokines such as IL-1, IL-2, and IL-12, can drive differentiation of T cells by influencing transcription factors necessary for promoting differentiation (Curtis et al. 1999). Collectively, these three signals cooperate together to coordinate signaling cascades that activate T cells and drive their proliferation and differentiation.

T cell proliferation and differentiation is tightly controlled. T cells not only coordinate immune responses to infection and tumors, but, when aberrantly activated or dysregulated, can cause chronic and destructive inflammation. Consequently, immune checkpoints and regulators of the immune system help to maintain immune homeostasis. A critical regulator of T cell activation and differentiation is transforming growth factor- $\beta$  (TGF- $\beta$ ). TGF- $\beta$  was first characterized due to its ability to induce a transformed phenotype in normal fibroblasts *in vitro*

(de Larco & Todaro 1978). TGF- $\beta$  has since been shown to influence a variety of cell types, regulating organ development, immune response, and cancer metastasis (Sanford et al. 1997; Sanderson et al. 1995).

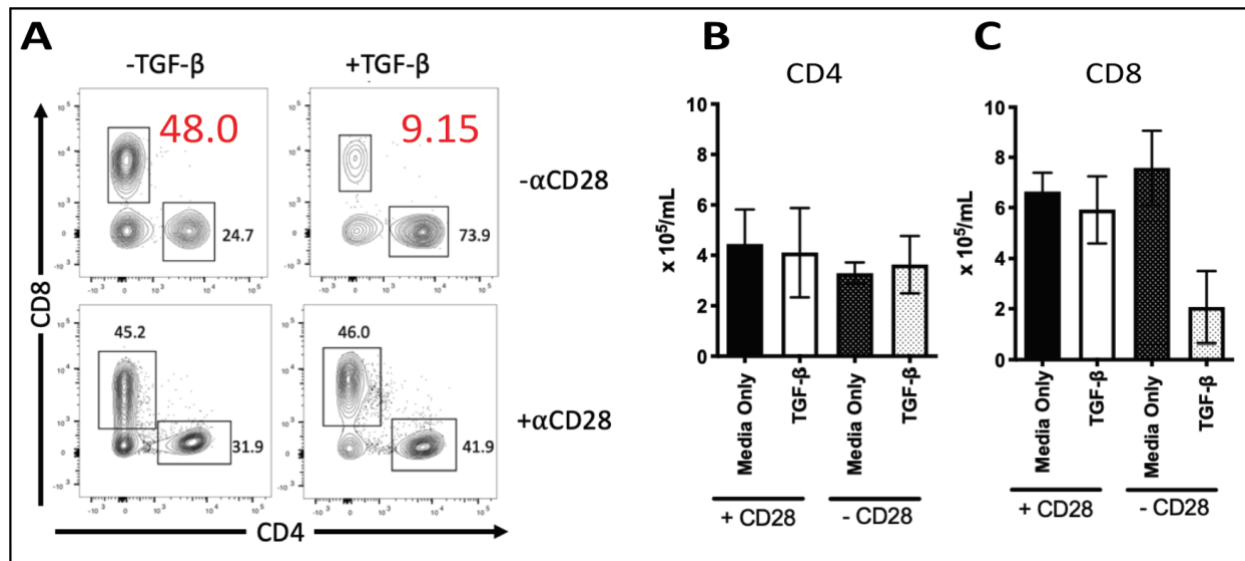
TGF- $\beta$  signaling acts on a number of pathways that influence cell growth and proliferation in multiple cell types. In T cells, TGF- $\beta$  is essential for maintaining immune homeostasis by regulating the development of CD4<sup>+</sup> effector T cell subsets, as well as contributing to the differentiation of naïve T cells into regulatory T cells (Tregs) by promoting the induction of Forkhead Box P3 (Foxp3), a transcription factor essential for Treg development (Gorelik, L. & Flavell, R.A. 2002; Liu, Y. et al. 2008). TGF- $\beta$  has also been shown to alter gene expression of other molecules such as a critical Ras activation factor, RasGRP1, essential for T cell activation (Takami et al. 2018).

To better understand the significance of TGF- $\beta$  signaling in T cells, our lab developed TGF- $\beta$  receptor I (TGF- $\beta$ RI) deficient Jurkat T cells. In the TGF- $\beta$ RI deficient Jurkat T cells, there is hyperactivation of c-Jun/Jun-B N-terminal kinase (JNK), suggesting that TGF- $\beta$  signaling is suppressing JNK activation in T cells. Additionally, in TGF- $\beta$ RI deficient Jurkat T cells, molecules upstream of JNK activation have increased expression, further suggesting that TGF- $\beta$  signaling suppresses T cell proliferation by modulating molecules of the JNK activation pathway. In contrast to CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cell responses to TGF- $\beta$  are not well characterized. TGF- $\beta$  inhibits CD8<sup>+</sup> T cell proliferation both *in vitro* and *in vivo*, but the molecular mechanism behind this inhibition is unknown.



### Suppressive Effects of TGF- $\beta$ on CD8<sup>+</sup> T cell Proliferation

We assessed the effects of TGF- $\beta$  signaling in primary murine T cells by stimulating total T cells with plate-bound anti-CD3 in the presence or absence of soluble anti-CD28 and TGF- $\beta$  (2.5 ng/mL) (Fig. 1). In the presence of anti-CD28 antibody, the addition of TGF- $\beta$  reduces the CD8<sup>+</sup> T cell frequency by 12% and increases the CD4<sup>+</sup> T cell frequency by 22% (Fig. 1A). Interestingly, in the absence of CD28 co-stimulation, the CD8<sup>+</sup> T cell frequency reduced by 38.85% while the CD4<sup>+</sup> T cell frequency increased by 49.2% (Fig. 1A). This frequency change was due to a reduction of the CD8<sup>+</sup> T cell number, while the total cell number of CD4<sup>+</sup> T cells did not change in the presence of TGF- $\beta$  (Fig. 1B and 1C). These data suggest that CD8<sup>+</sup> T cell proliferation is highly sensitive to TGF- $\beta$  in the absence of CD28 co-stimulation.

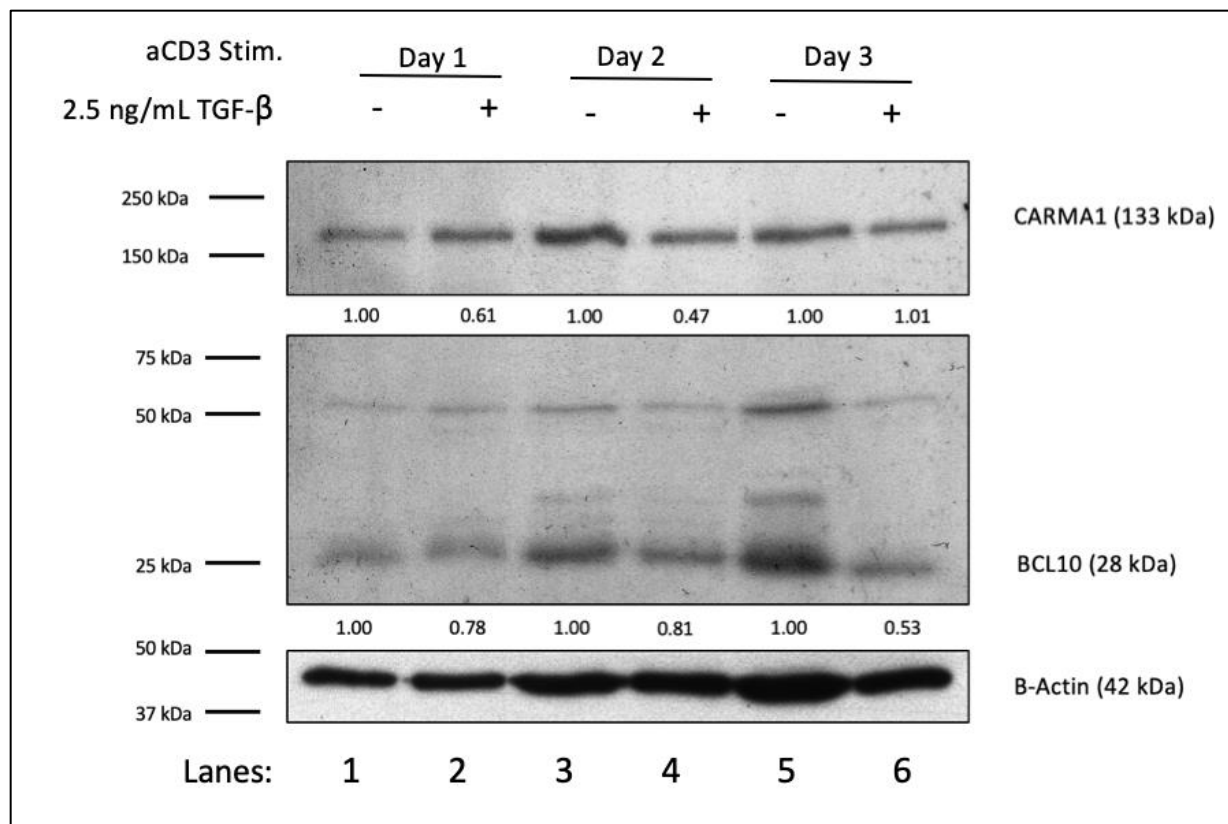


**Figure 1. Murine CD4<sup>+</sup> and CD8<sup>+</sup> T cell Proliferation in the Presence or Absence of TGF- $\beta$**  Splenic T cells purified by panning using goat anti-mouse IgG and IgM antibodies were stimulated by plate-bound anti-CD3 (A)  $\pm$  soluble anti-CD28 and  $\pm$  2.5 ng/mL TGF- $\beta$ . After 3 days of stimulation, cells were stained for CD4 and CD8 and analyzed by flow cytometry. (B) CD4<sup>+</sup> and (C) CD8<sup>+</sup> cell counts were determined by using the frequency of CD4<sup>+</sup> and CD8<sup>+</sup> cells analyzed by flow and multiplying the frequency by the cell count of each well. Bar graphs consist of counts from three independent experiments.

Our previous data highlights how TGF- $\beta$  signaling suppresses activation of JNK. TGF- $\beta$ RI deficient Jurkat T cells are highly sensitive to TCR stimulation and show constitutively activated JNK (Jacks et al. 2019 *in progress*). JNK activation is critical for T cell activation, proliferation, and differentiation (Dumont et al. 1998; Xia et al. 1995; Yang et al. 1998). Therefore, we hypothesized that in murine primary T cells, the JNK activation pathway is modulated by TGF- $\beta$  signaling.

Paradoxically, previous work by others demonstrate that JNK can be activated by transforming growth factor- $\beta$  activating kinase 1 (TAK1), which is required for TGF- $\beta$  induced transcriptional regulation (Wang et al. 2001). Our data indicate that the loss of TGF- $\beta$  signaling causes hyperactivation of JNK, suggesting that in T cells, TGF- $\beta$  has an inverse effect on the JNK pathway.

To test whether TGF- $\beta$  signaling suppresses CD8<sup>+</sup> T cell proliferation through inhibition of CARMA1-BCL10-MALT1 (CBM) complex formation, we detected the expression of the CBM complex by western blot in plate-bound anti-CD3 stimulated T cells. Over three days of stimulation, CARMA1 and BCL10 steadily increase in expression (Fig. 2 lanes 1, 3, and 5). In the presence of TGF- $\beta$ , CARMA1 and BCL10 did not increase and remained at similar levels over the three days of stimulation (Fig. 2 lanes 2, 4, and 6). Together these data suggest that TGF- $\beta$  signaling inhibits CARMA1 and BCL10 expression in T cells. We will further define the mechanisms by which TGF- $\beta$  inhibits CBM complex expression.



**Figure 2. CARMA1 and BCL10 Expression in the Presence or Absence of TGF-β** Splenic T cells purified by panning using goat anti-mouse IgG and IgM antibodies were stimulated by plate-bound anti-CD3 antibody in the presence or absence of 2.5 ng/mL TGF-β. After 3 days of stimulation, cells were lysed by SDS and analyzed by Western Blot. Relative intensity of each band determined by ImageJ analysis (shown below each lane) was determined against day 0 after standardizing to β-actin.

### CD25 Expression is Reduced by TGF-β

Previous work has shown that TGF-β reduces CD25 expression on antigen stimulated T cells to limit IL-2 signaling to control proliferation (Tiemessen et al. 2004). Due to these findings, we hypothesized that TGF-β reduced CD25 expression, suppressing proliferation and subsequent expression of signaling molecules necessary for T cell activation and proliferation.

We wanted to determine when CD25 expression was reduced by TGF-β. CD25 is the high affinity component of the IL-2 receptor, necessary for adequate IL-2 recognition and

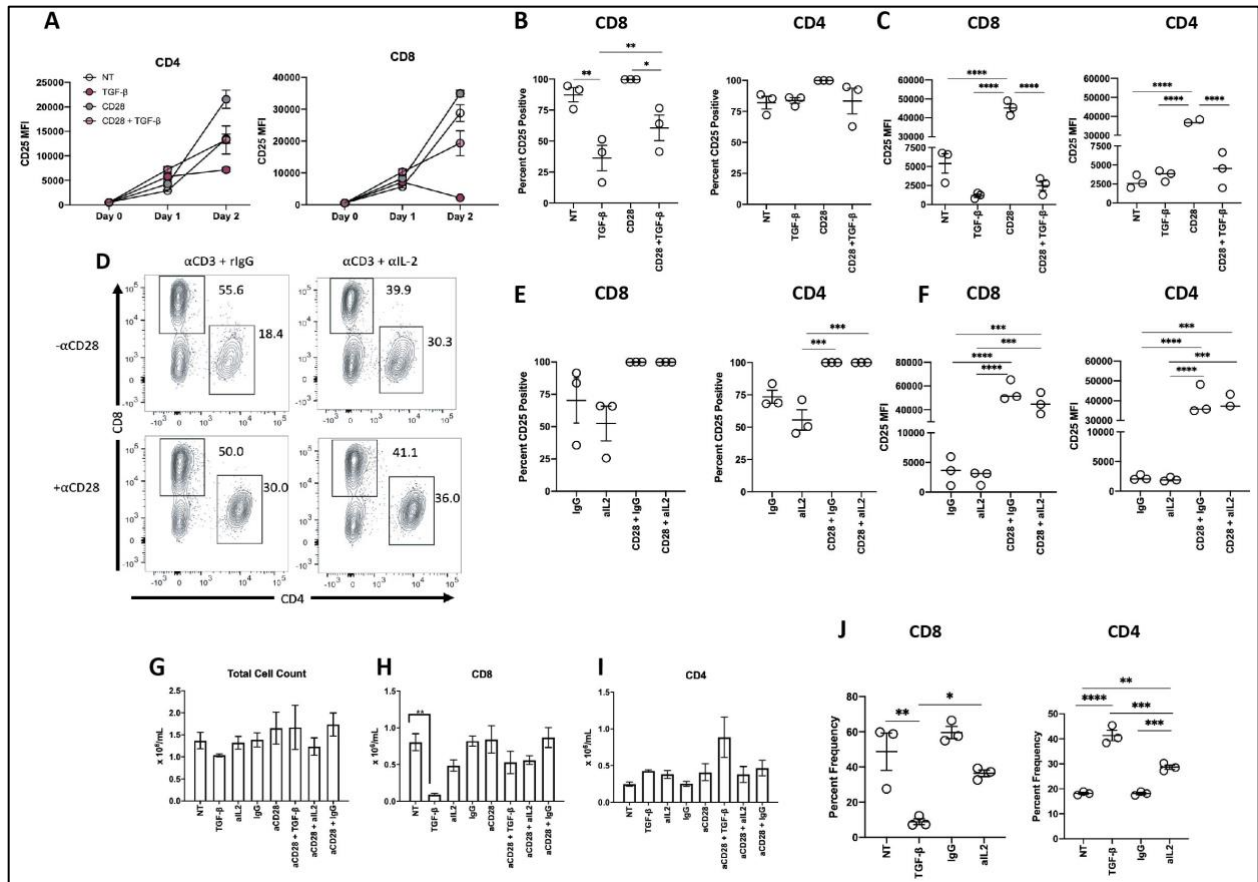
downstream signaling. We hypothesized that TGF- $\beta$  would reduce CD25 expression upon activation by anti-CD3 and/or anti-CD28. To test this hypothesis, we stimulated total splenocytes by plate-bound anti-CD3  $\pm$  soluble anti-CD28 and TGF- $\beta$  for up to two days. After indicated days of stimulation, CD25 surface expression was determined by flow cytometry. We found that after one day of stimulation, all conditions upregulated CD25 surface expression to similar levels, however after two days of stimulation, TGF- $\beta$  reduces both the MFI (Fig. 3A) and percent (data not shown) of cells positive for CD25 in CD4+ and CD8+ T cell populations.

We further characterized CD25 expression after three days of stimulation by flow cytometry. As previously shown, TGF- $\beta$  reduces the CD8+ T cell frequency and total cell number in the absence of CD28 co-stimulation. Here we show that decreased CD25 expression correlates with reduced proliferation of CD8+ T cells. After three days of stimulation, where we see the greatest decrease in CD8+ T cell frequency in the presence of TGF- $\beta$ , there is also a dramatic decrease in CD25 surface expression when compared to the absence of TGF- $\beta$  (Fig. 3B and 3C). TGF- $\beta$  did not have the same suppressive effect on CD4+ T cell frequency and cell number as CD8+ T cells (Fig. 1A) and did not reduce CD25 expression as dramatically in CD4+ T cells as CD8+ T cells (Fig. 3B and 3C).

We observed that anti-CD3 stimulation is sufficient in upregulating CD25 surface expression in both CD4+ and CD8+ T cell subsets (Fig. 3B and 3C). The addition of TGF- $\beta$  causes a slight increase in CD25 expression in CD4+ T cells, but the majority of CD8+ T cells are CD25 negative. Additionally, CD28 co-stimulation restores CD25 surface expression, confirming our hypothesis that TGF- $\beta$  reduces CD25 expression, suggesting that CD25 reduction suppresses T cell proliferation.

IL-2 production and CD25 expression is upregulated upon TCR activation, and CD25 expression is sustained by IL-2 signaling, forming a feedforward loop between IL-2 production and CD25 expression (). Therefore, it is a possibility that TGF- $\beta$  is reducing CD25 expression by blocking IL-2 production. We next determined if blocking IL-2 signaling by neutralizing IL-2 using an anti-IL2 antibody would suppress CD8 T cell growth and CD25 expression.

To test this hypothesis, we stimulated total splenocytes by plate-bound anti-CD3 as before,  $\pm$  neutralizing anti-IL-2 antibody and soluble anti-CD28 (Fig. 3D). After three days of stimulation, CD25 surface expression was analyzed. In the presence of anti-IL-2 neutralizing antibody, both CD4+ and CD8+ T cell populations have a decrease in percent CD25 positive cells and CD25 MFI (Fig. 3E and 3F). Additionally, TGF- $\beta$  dramatically reduces the total cell number and frequency of CD8+ T cells, however this is not recapitulated by the neutralization of IL-2 (Fig. 3H and 3J). Furthermore, neutralizing IL-2 reduces both CD4 and CD8 population frequencies, confirming that the anti-IL-2 neutralizing antibody blocks IL-2 signaling resulting in both CD4 and CD8 frequency and proliferation (Fig. 3H—J). From this data, we can conclude that the reduction in CD25 expression on CD8+ T cells is not solely due to reduced IL-2 production of T cells in the presence of TGF- $\beta$ . This is also in part due to IL-2 being a necessary growth factor for both CD4+ and CD8+ T cells. It also begins to elucidate a cell intrinsic difference between CD4+ and CD8+ T cells, where CD8+ T cells are much more sensitive to TGF- $\beta$  when CD28 co-stimulation is not provided.

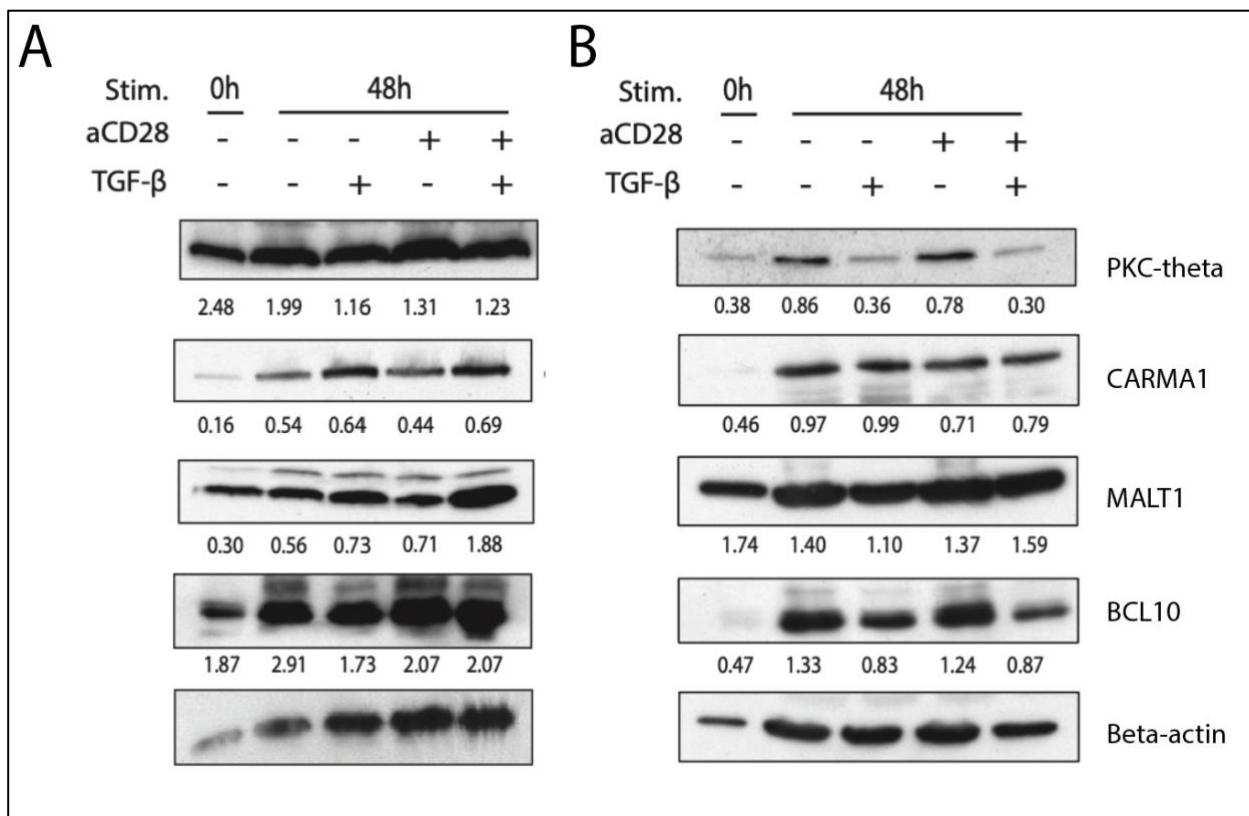


**Figure 3. CD25 Expression is Decreased by TGF-β** (A) Total splenocytes were stimulated by plate-bound anti-CD3 for indicated duration  $\pm$  soluble anti-CD28 and/or 2.5 ng/mL TGF- $\beta$ ; cells were analyzed by flow cytometry and kinetics of CD25 MFI was determined. (B) Percent positive, and (C) MFI of CD25 expression after three days of stimulation. (D) Total splenocytes were stimulated by plate-bound anti-CD3 for three days  $\pm$  soluble anti-CD28 and/or 10 ng/mL anti-IL2 neutralizing antibody or rat IgG isotype control; cells were analyzed by flow cytometry and CD4 and CD8 population frequencies were determined. (E) Percent positive, and (F) MFI of CD25 expression after three days of stimulation. Statistical analysis (B, C, E, F) by Ordinary one-way ANOVA Tukey's multiple comparisons test (\* $p \leq 0.039$ , \*\* $p \leq 0.0019$ , \*\*\* $p \leq 0.0004$ , \*\*\*\* $p \leq 0.0001$ ), bars represent mean and standard error of the mean (SEM). (G) Total cell count determined by trypan blue stain. (H) CD8 and (I) CD4 cell count determined by flow cytometry frequencies and multiplying by total cell count. Statistical analysis (G—I) by Ordinary one-way ANOVA Tukey's multiple comparisons test (\*\* $p \leq 0.0025$ ), bars represent mean and standard error of the mean (SEM). (J) Percent CD8<sup>+</sup> and CD4<sup>+</sup> determined from flow cytometry analysis. Statistical analysis (J) by Ordinary one-way ANOVA Tukey's multiple comparisons test (\*\*\*\* $p \leq 0.0001$ , \*\*\* $p \leq 0.0003$ , \*\* $p \leq 0.001$ , \* $p \leq 0.038$ ).

### CARMA1-BCL10-MALT1 Complex Expression is Restricted by TGF-β in CD8<sup>+</sup> T cells

We sought to further elucidated the difference between CD4 and CD8 T cell proliferate status in the presence of TGF-β. Previously, we observed a decrease in the CBM complex in a

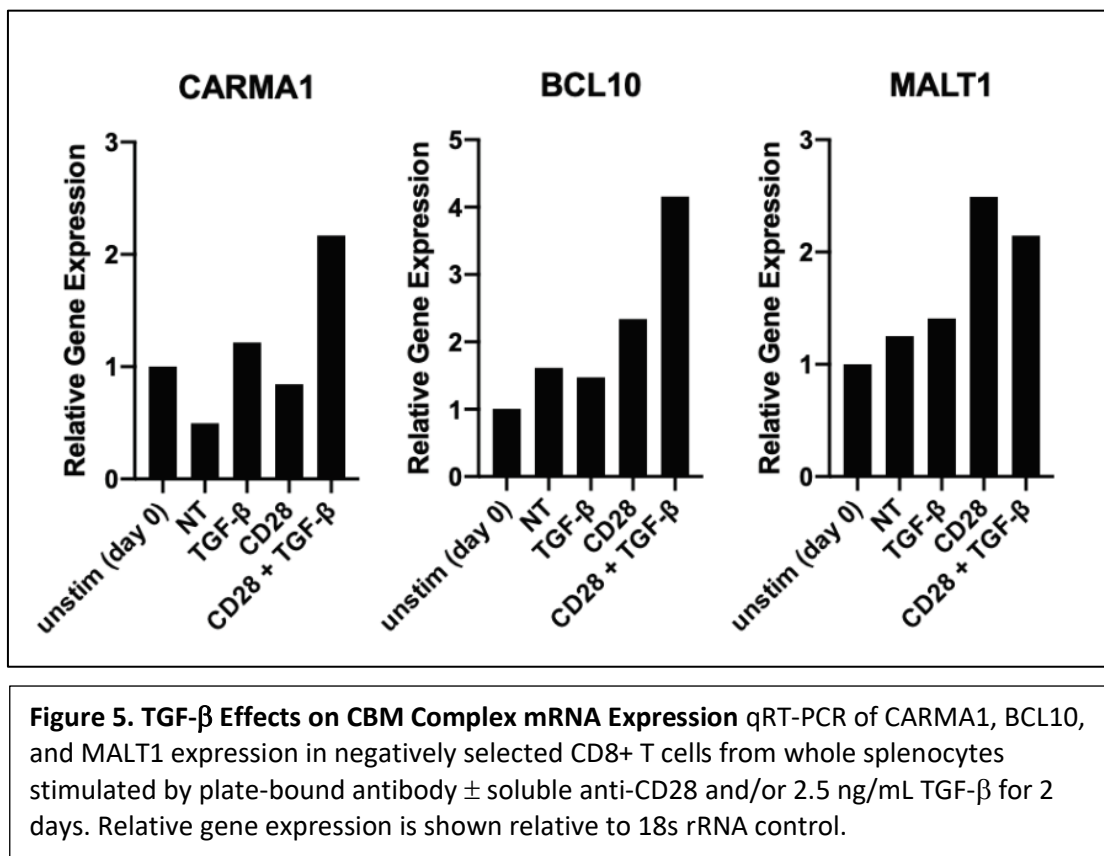
mixed population of CD4<sup>+</sup> and CD8<sup>+</sup> T cells and hypothesized that expression would be further reduced in isolated CD8<sup>+</sup> T cells. To test this hypothesis, we isolated either CD4<sup>+</sup> or CD8<sup>+</sup> T cells by negative selection and stimulated purified CD4<sup>+</sup> or CD8<sup>+</sup> T cells by plate-bound anti-CD3 in the presence or absence of soluble anti-CD28 and/or TGF- $\beta$  for 48 hours. TGF- $\beta$  has little to no effect on PKC- $\theta$ , CARMA1, MALT1, or BCL10 expression in CD4<sup>+</sup> T cells (Fig. 4A), however in CD8<sup>+</sup> T cells, their expression is greatly reduced (Fig. 4B). Furthermore, in CD8<sup>+</sup> T cells, CD28 co-stimulation fails to restore expression of PKC- $\theta$  and BCL10 in TGF- $\beta$  suppressed T cells (Fig. 4B). Together this data suggests that CD4<sup>+</sup> and CD8<sup>+</sup> T cells have cell intrinsic differences in their response to TGF- $\beta$ .



**Figure 4. CD8+ T cell CBM Expression in the Presence of TGF- $\beta$**  Total CD4+ (A) or CD8+ (B) T cells were isolated by negative selection from whole splenocytes and stimulated by plate-bound anti-CD3 antibody  $\pm$  soluble anti-CD28 and/or 2.5 ng/mL TGF- $\beta$  for 2 days. Cells were lysed by SDS and analyzed by Western Blot. Relative intensity of each band determined by ImageJ analysis (shown below each lane) was determined against  $\beta$ -actin loading control.

To address whether the decrease in CBM complex expression due to TGF- $\beta$  was controlled at the transcriptional or translational level, we analyzed mRNA expression of CARMA1, BCL10, and MALT1. As previously described, CD8+ T cells were isolated by negative selection and then stimulated for two days. After stimulation, mRNA was isolated and relative gene expression was determined by qRT-PCR. In this preliminary experiment, there is no decrease in mRNA expression of CBM complex molecules in the presence of TGF- $\beta$  (Fig. 5). This data suggests that TGF- $\beta$  signaling is not restricting transcription of these molecules.





## Discussion

We have identified a CD8<sup>+</sup> T cell intrinsic difference in the response to TGF- $\beta$  signaling compared to the CD4<sup>+</sup> T cell response. The CD4<sup>+</sup> versus CD8<sup>+</sup> T cell response to TCR stimulation alone (no CD28 co-stimulation) in the presence of TGF- $\beta$  reveals a suppressive effect of activation pathways downstream of TCR signaling. CD4<sup>+</sup> and CD8<sup>+</sup> T cells differ in their priming and subsequent proliferation after antigen stimulation, thus the effect of TGF- $\beta$  on signaling complexes downstream of TCR activation in CD8<sup>+</sup> T cells but not CD4<sup>+</sup> T cells at the indicated time point could be due to a difference in activation kinetics between subsets. Expression of early activation molecules, such as PLC-gamma, LAT, and GRB2 (data not shown), as well as PKC- $\theta$  and downstream signaling molecules are restricted by TGF- $\beta$ . The activation of PKC- $\theta$  by

CD28 co-stimulation could be a critical crosstalk mediating CD8<sup>+</sup> T cell survival in TGF- $\beta$  suppressed conditions. Further experiments will need to be done to elucidate the effects of TGF- $\beta$  signaling on the activation and expression of PKC- $\theta$  in the presence or absence of CD28 co-stimulation.

We hypothesize that this restricted expression results in diminished activation status after stimulation as determined by a decrease in CD25 expression and subsequent decrease in frequency and proliferation.

We hypothesize two potential mechanisms of regulation: (1) Smad mediated transcriptional repression/recruitment to affected genes, and/or (2) MicroRNA mediated post-transcriptional repression of affected genes.

IL-2 is a critical growth factor for T cell proliferation. The IL-2 receptor is a trimer of the common gamma chain ( $\gamma_c$ ), CD122, and CD25, the high affinity alpha subunit. Upon T cell activation (TCR stimulation), IL-2 is produced and CD25 expression is upregulated, creating a feed forward loop. Additionally, IL-2 and IL-15 share a number of biological activities, both  $\gamma_c$  and CD122 receptors, and the downstream signaling molecule STAT5. IL-15 is known to be a critical cytokine for T and natural killer cell proliferation. IL-15 has also been shown to be a regulator of CD8<sup>+</sup> memory T cell development, indicating a pro-survival role of IL-15 in CD8<sup>+</sup> T cell function. The role of IL-15 and its alpha receptor subunit (IL-15R $\alpha$ ) should also be explored to better understand how different T cell subsets respond to TGF- $\beta$ .

TGF- $\beta$  is well characterized as an inducer of epithelial to mesenchymal transition (EMT). In addition to Smad-mediated transcription of EMT markers, TGF- $\beta$  signaling also induces microRNA expression, a critical component in the overall signaling cascade during EMT. Due to

the induction of microRNAs by TGF- $\beta$ , it is feasible that microRNA expression is mediating the suppression of signaling molecules in CD8<sup>+</sup> T cells.

## CHAPTER FOUR

### THE ROLE OF INTERLEUKIN (IL)-4 IN RESTORING PROLIFERATION TO TGF- $\beta$ SUPPRESSED CD8<sup>+</sup> T CELLS

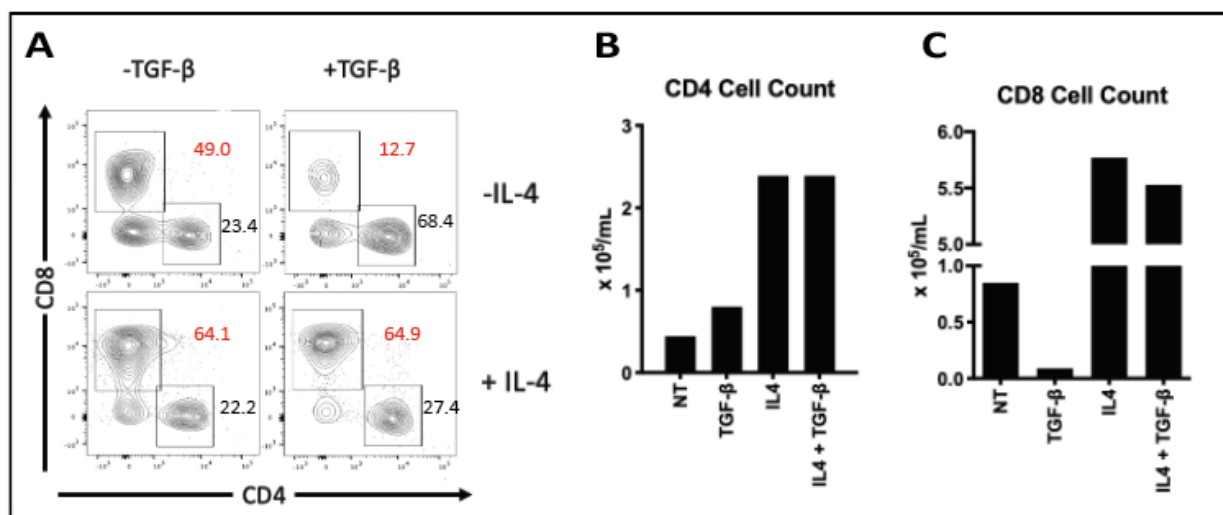
#### Introduction

Our previous work has shown that the addition of IL-4 blocks Treg differentiation, suggesting IL-4 is counteracting the effects of TGF- $\beta$  signaling in T cells. IL-4 is known to drive CD4<sup>+</sup> T cell differentiation towards Th2 effector T cells, but its role in CD8<sup>+</sup> T cell activation is not well characterized. We have found that IL-4 reverses TGF- $\beta$ -mediated suppression in CD8<sup>+</sup> T cells as well as enhance anti-tumor function of CD8<sup>+</sup> T cells. In this aim, we will determine how IL-4 restores CD8<sup>+</sup> T cell proliferation and whether IL-4 can promote anti-tumor function of CD8<sup>+</sup> T cells *in vivo*.

Our previous work demonstrates that T cells from human umbilical cord blood (UCB) have an increased predisposition to differentiate into Tregs compared to T cells from adult blood in a TGF- $\beta$  dependent manner (Lee, J. G. 2016). Furthermore, the addition of IL-4 was shown to block Treg generation in the UCB Treg differentiation conditions, suggesting that IL-4 is counteracting the effects of TGF- $\beta$  in UCB Treg differentiation. We hypothesize that IL-4 counteracts TGF- $\beta$  mediated suppression of CD8<sup>+</sup> T cells.

### Effect of IL-4 on TGF- $\beta$ Suppressed CD8<sup>+</sup> T cells

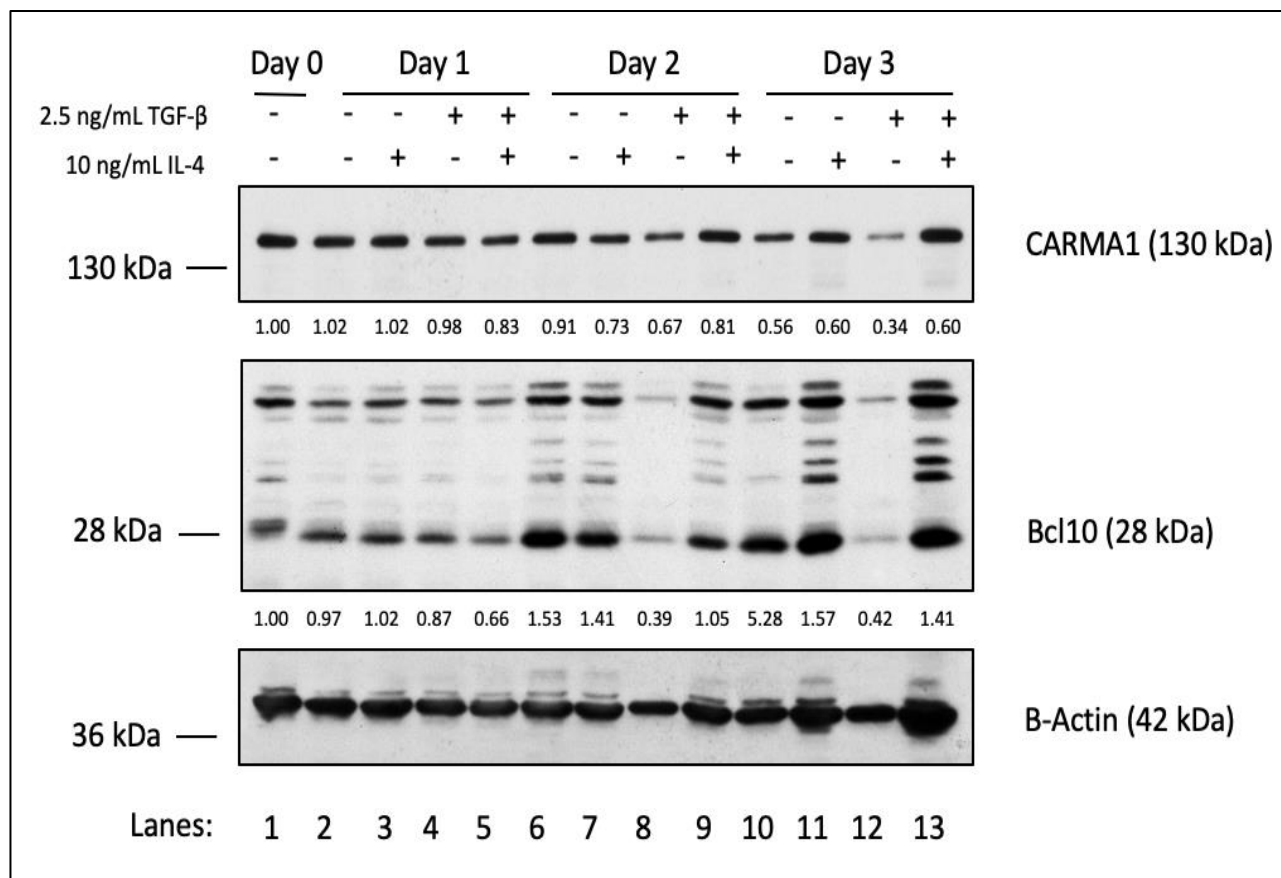
We assessed the effects of IL-4 in TGF- $\beta$ -mediated suppressed CD8<sup>+</sup> T cells in primary murine T cells by stimulating T cells with plate-bound anti-CD3 in the presence or absence of TGF- $\beta$  (2.5 ng/mL) and the presence or absence of IL-4 (10 ng/mL) (Fig. 6A). In the presence of TGF- $\beta$ , the CD8<sup>+</sup> T cell frequency decreases by 34% while the CD4<sup>+</sup> T cell frequency increases by 44.9% (Fig. 6A). In the presence of IL-4, CD8<sup>+</sup> T cell frequency increase by 17%, and remain at a similar frequency in the presence of both TGF- $\beta$  and IL-4 (Fig. 6A). The CD4<sup>+</sup> T cell number is not reduced by TGF- $\beta$  (Fig. 6B) while the CD8<sup>+</sup> T cell number is greatly reduced by TGF- $\beta$  (Fig. 6C). Interestingly, there is a greater cell number for both CD4<sup>+</sup> and CD8<sup>+</sup> T cells with IL-4 and remains at a similar level in the presence of both TGF- $\beta$  and IL-4 in CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Fig. 6B and 6C). These data suggest that TGF- $\beta$ -mediated suppressed CD8<sup>+</sup> T cell proliferation is restored by IL-4.



**Figure 6. Murine CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the Presence or Absence of TGF- $\beta$  and/or IL-4** Splenic T cells purified by panning using goat anti-mouse IgG and IgM antibodies were stimulated by plate-bound anti-CD3 antibody in the presence or absence of 2.5 ng/mL TGF- $\beta$  and/or 10 ng/mL IL-4. After 3 days of stimulation, cells were stained for CD4 and CD8 and analyzed by flow cytometry (A). (B) CD4<sup>+</sup> and (C) CD8<sup>+</sup> cell counts were determined by using the frequency of CD4<sup>+</sup> and CD8<sup>+</sup> cells analyzed by flow and multiplying the frequency by the cell count of each well.

### **IL-4 Effect on the CARMA1-BCL10-MALT1 Complex**

Next, we tested whether the effect of IL-4 on CD8<sup>+</sup> T cell proliferation is due to an increase in the CBM complex. CARMA1 expression is reduced by day three of stimulation in the presence of TGF- $\beta$  (Fig. 7 lane 12), however expression is restored in the presence of both TGF- $\beta$  and IL-4 (Fig. 7 lane 13). BCL10 expression is also reduced after two days of stimulation in the presence of TGF- $\beta$  (Fig. 7 lane 8) and this reduction is maintained after day three of stimulation (Fig. 7 lane 12). Furthermore, the addition of IL-4 and TGF- $\beta$  result in restored expression of BCL10 after two and three days of stimulation (Fig. 7 lanes 9 and 13). Together this data suggests that IL-4 is restoring proliferation to TGF- $\beta$  mediated suppressed CD8<sup>+</sup> T cells by restoring expression of CARMA1 and BCL10.



**Figure 7. CARMA1 and BCL10 Expression in the Presence or Absence of TGF-β and/or IL-4** Splenic T cells purified by panning using goat anti-mouse IgG and IgM antibodies were stimulated by plate-bound anti-CD3 antibody in the presence or absence of 2.5 ng/mL TGF-β and/or 10 ng/mL IL-4. After each day of stimulation, cells were lysed by SDS and analyzed by Western Blot. Relative intensity of each band determined by ImageJ analysis (shown below each lane) was determined against day 0 after standardizing to β-actin.

## Discussion

Our data show that IL-4 abrogates TGF-β induced expression of Foxp3 by CD4<sup>+</sup> T cells. We have also shown that IL-4 restores CD8<sup>+</sup> T cell frequency and proliferation as well as CBM complex expression to TGF-β suppressed T cells. Together, this data suggests a pro-survival role of IL-4 to TGF-β suppressed T cells.

IL-4 regulates Th2 cell differentiation and function as well as B cell activation and survival. It is commonly associated with allergy and asthmatic inflammation, however, has also been shown to generate CD8<sup>+</sup> memory T cells (Renkema et al. 2016). In the tumor microenvironment, Tregs are immune suppressive and block antitumor function of cytotoxic T cells through their cytokine production, as well as consumers of IL-2. One drawback of using IL-2 as an immunotherapy is its effect on regulatory T (Treg) cells. We have previously shown that IL-4 blocks the induction of Foxp3 expression in CD4<sup>+</sup> T cells, a critical transcription factor in Treg differentiation and function. Furthermore, IL-4 was shown to restore proliferation to CD8<sup>+</sup> cytotoxic T cells, suggesting that IL-4 could be a potential cytokine used for immunotherapy as it both blocks Treg differentiation and induces cytotoxic T cell proliferation.

Our data demonstrates that heat-killed B16 melanoma cells inhibit antigen-receptor induced CD8<sup>+</sup> T cell proliferation in a TGF- $\beta$  dependent manner. If CD8<sup>+</sup> T cell proliferation is enhanced by IL-4, then we will test if IL-4 also augments CD8<sup>+</sup> T cell proliferation in the presence of heat-killed B16 cells. We will also test if IL-4 restores inflammatory cytokine production to CD8<sup>+</sup> T cells suppressed by TGF- $\beta$ . Finally, to study if IL-4 restores CD8<sup>+</sup> effector function *in vivo*, we will generate IL-4 producing melanoma cells and determine whether B16 specific CD8<sup>+</sup> T cells have increased anti-tumor function against IL-4 producing B16 melanoma tumors.



## CHAPTER FIVE

### GENERAL DISCUSSION

Our data demonstrates a cell intrinsic difference in response to TGF- $\beta$  in CD8+ versus CD4+ T cells. Upon TCR stimulation in the presence of TGF- $\beta$ , the CD8+ T cell response downstream of TCR activation is suppressed, therefore restricting adequate activation of necessary pathways and transcription factors for efficient T cell function and survival. We have also observed that CD28 co-stimulation can overcome TGF- $\beta$  mediated suppression, indicating a crosstalk between TCR, CD28, and TGF- $\beta$  signaling. Suppression of cytotoxic CD8+ T cell proliferation and function is often observed in solid tumors expressing high levels of TGF- $\beta$ , therefore elucidating the molecular mechanism behind TGF- $\beta$  mediated suppression could reveal a potential pathway or molecule for targeted therapy in tumors with elevated TGF- $\beta$  levels.

The high affinity IL-2 receptor alpha subunit (CD25) surface expression is decreased by TGF- $\beta$  in CD8+ compared to TCR stimulation alone. Alternatively, CD4+ T cells did show reduced CD25 expression overall, however a population of CD4+ T cells stimulated in the presence of TGF- $\beta$  remained high expressors of CD25. It is probable that this CD4+CD25-high population is Foxp3+, resulting in the early generation of induced Tregs or maintenance of preexisting Tregs, however this would need to be confirmed in future experiments. The expression of Foxp3 in

CD8<sup>+</sup> T cells activated in the presence of TGF- $\beta$  could also contribute to the differing response between CD4<sup>+</sup> and CD8<sup>+</sup> T cells.

We further began elucidating the molecular mechanism of TGF- $\beta$  mediated suppression resulting in decreased CD25 expression and subsequent T cell proliferation. The IL-2 receptor alpha (CD25) gene is primarily controlled by transcription factors, NFAT, AP-1, and NF- $\kappa$ B, upregulated upon TCR stimulation. We found that PKC- $\theta$  and BCL10, two CBM signalosome proteins necessary for NF- $\kappa$ B activation, were restricted by TGF- $\beta$  in CD8<sup>+</sup> but not CD4<sup>+</sup> T cells. These data suggest that controlling activation and expression of the CBM signalosome complex could result in diminished NF- $\kappa$ B nuclear translocation and subsequent decreased expression of target genes such as CD25. Future chromatin immunoprecipitation (ChIP) experiments to address NF- $\kappa$ B binding to the CD25 promoter region will determine whether NF- $\kappa$ B suppression is mediating the decrease in CD25 expression in CD8<sup>+</sup> T cells. Additionally, the activation of PKC- $\theta$  by CD28 co-stimulation could be a critical crosstalk mediating CD8<sup>+</sup> T cell survival, therefore further experiments will need to be done to elucidate the effects of TGF- $\beta$  signaling on the activation and expression of PKC- $\theta$  in the presence or absence of CD28 co-stimulation in CD8<sup>+</sup> T cells.

These data together raise the question of how TGF- $\beta$  targets a number of molecules downstream of TCR and IL-2 signaling. In epithelial cells, TGF- $\beta$  stimulates microRNA expression that has an overall broad effect on cellular responses and induces epithelial to mesenchymal transition (EMT). TGF- $\beta$  induced EMT plays a fundamental role in embryonic development, wound healing, tissue regeneration, organ fibrosis, and cancer metastasis (Carew, Wang, Kantharidis 2012). More specifically, the micro-RNA family miR-200 has been shown to regulate

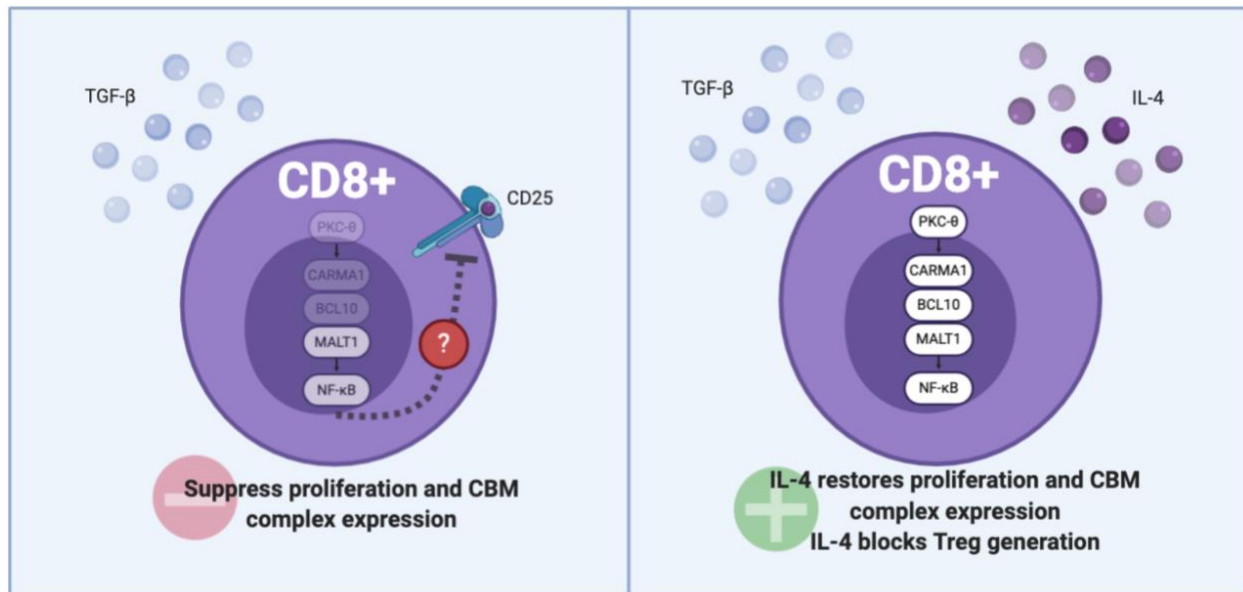
T cell fate into memory CD8<sup>+</sup> T cells by controlling expression of transcription factors ZEB1 and ZEB2 (Guan et al. 2018). It would be plausible that the miR-200 family is responsible for regulating expression of targeted molecules observed in our system.

Lastly, we established that IL-4 could counteract the suppressive effects of TGF- $\beta$ . Previous studies in our lab have shown the abrogation of TGF- $\beta$  induced expression of Foxp3 in CD4<sup>+</sup> T cells, impeding Treg induction. In CD8<sup>+</sup> T cells, IL-4 has been shown to induce a memory T cell phenotype, suggesting a pro-survival role of IL-4 in CD8<sup>+</sup> T cell function (Renkema et al. 2016). Together with our data, this suggests that IL-4 counteracts TGF- $\beta$  signaling, resulting in increased CD8<sup>+</sup> T cell survival.

One consequence of using IL-2 as immunotherapy is the support of Treg survival and function, resulting in a more suppressive tumor microenvironment. We have shown that IL-4 can counteract TGF- $\beta$  mediated suppression in addition to blocking the generation of Tregs. Together, these data suggest that IL-4 or an IL-4 analogue could potentially be used as an immunotherapy to augment the CD8 cytotoxic T cell response.

In conclusion, we have built a model of the possible mechanism behind TGF- $\beta$  mediated suppression of CD8<sup>+</sup> T cells (Fig. 8). We have shown that TGF- $\beta$  downregulates the expression of necessary molecules upstream of NF- $\kappa$ B and JNK pathway activation, suggesting a decrease in NF- $\kappa$ B and AP-1 activation and subsequent diminished target gene activation. We hypothesize that one of these target genes is CD25, the high affinity IL-2 receptor. We have shown that TGF- $\beta$  reduces CD25 expression on CD8<sup>+</sup> T cells and future experiments will address the promoter activity of the CD25 gene in the presence of TGF- $\beta$ . Lastly, we observe that IL-4 counteracts the suppressive effects of TGF- $\beta$  on CD8<sup>+</sup> T cells by restoring expression of

CARMA1 and BCL10, suggesting an increase in NF- $\kappa$ B transcription factor activity. This indicates that IL-4 supports the proliferation of CD8<sup>+</sup> T cells and could be a potential immunotherapy candidate due to its further impeding effects on Treg induction.



**Figure 8. Model of TGF- $\beta$  Mediated Suppression and the Effects of IL-4 on TGF- $\beta$  Suppressed CD8<sup>+</sup> T cells** TGF- $\beta$  reduces PKC- $\theta$  and CBM complex expression, resulting in suppressed proliferation and CD25 expression in CD8<sup>+</sup> T cells. IL-4 counteracts the suppressive effects of TGF- $\beta$  in CD8<sup>+</sup> T cells, restoring proliferation to TGF- $\beta$  suppressed cells.

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## VITA

Shena J. Geisinger was born in Seoul, Korea, April 20<sup>th</sup>, 1996, and adopted by Eric and Ann Geisinger. She attended Grand View University In Des Moines, Iowa, where she double majored and earned her Bachelor's of Science in Biochemistry and Bachelor's of Arts in Biology. While at Grand View, Shena began her research experience under the mentorship of Dr. Bonnie Hall, studying the function of the PETase enzyme, and Dr. Adriana LaGier. studying the effects of vibration on epithelial to mesenchymal transition (EMT).

In 2018, Shena matriculated in the Biochemistry and Molecular Biology program at Loyola. Soon after, Shena joined the lab of Dr. Makio Iwashima where she studied the suppressive effects of TGF- $\beta$  on CD8+ T cell proliferation.

